G-proteins coupled to phosphoinositide hydrolysis in the cochlear and vestibular sensory epithelia of the rat are insensitive to cholera and pertussis toxins

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Abstract

In the cochlear (CSE) and vestibular sensory epithelia (VSE), phosphoinositides are hydrolyzed in response to stimulation of phospholipase C (PLC) by cholinergic muscarinic and purinergic P2y agonists. Such receptor-mediated activation of PLC is expected to be coupled through guanine nucleotide-binding proteins (G-proteins). Although several classes of G-proteins have been identified in the inner ear, nothing is known about the type of G-proteins associated with the phosphoinositide second messenger system in CSE and VSE. Phosphoinositide hydrolysis was determined by the release of radiolabeled inositol phosphates (InsPs). Ten mM NaF plus 10 μM AlCl3 increased basal InsPs accumulation 2-fold in both CSE and VSE of the rat. Release of InsPs was also enhanced by guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) in saponin-permeabilized tissues. Furthermore, release of InsPs stimulated by both carbamylcholine (CCh) and adenosine 5'-O-[3-thiotriphosphate] (ATP-β-S) was significantly inhibited by 100 μM guanosine 5'-O-[2-thiodiphosphate] (GDP-β-S). These results strongly suggest the involvement of G-proteins in the receptor-PLC coupling in CSE and VSE. ADP-ribosylation in membrane fractions of CSE and VSE in the presence of cholera toxin (CTX) or pertussis toxin (PTX) indicated the existence of Gα- and Gβ-type G-proteins. However, neither CTX nor PTX affected basal or agonist-stimulated release of InsPs. These observations suggest that muscarinic and P2y purinergic receptors are coupled to PLC via CTX- and PTX-insensitive G-proteins in CSE and VSE.

Key words: G-protein; Inositol phosphate; ADP-ribosylation; Cholera toxin; Pertussis toxin; Cochlea; Vestibule; Rat

1. Introduction

Guanine nucleotide-binding proteins (G-proteins) couple plasma membrane receptors to their effector enzymes in several signal transduction systems. Most notably, specific members of this protein 'superfamily' mediate the hormonal stimulation of adenylate cyclase (Gα), phospholipase C (Gp; also designated Gq), and cyclic GMP-dependent phosphodiesterase (Gγ); the inhibition of adenylate cyclase (Gi); or are implicated in the regulation of ion channels (G, Go). G-proteins are heterotrimers consisting of α, β and γ subunits; α subunits have specific structural elements and distinguish different G-proteins, while the amino acid sequences of β and γ subunits are more conserved. G-proteins are activated by the receptor-catalyzed exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the α subunit, and inactivated by the subsequent hydrolysis of GTP to GDP. While the α subunit has long been recognized as an allosteric effector of its target enzymes, recent evidence indicates that β and γ subunits also affect enzyme activity (for reviews see Sternweis and Smrcka, 1992; Hepler and Gilman, 1992).

G-proteins can be classified by their differential sensitivity to cholera (CTX) and pertussis (PTX) toxins. These toxins catalyze the ADP-ribosylation of specific α-subunits leading to activation of Gq by CTX or to inactivation of Gi, Go and Gp by PTX (Gill and Meren, 1978; Cassel and Pfeuffer, 1978; Katada and Ui, 1982; see also Gilman, 1987, for review). In contrast, G-proteins which couple muscarinic receptors to the phosphoinositide second messenger system (Gp) are mostly insensitive to CTX or PTX action (Masters et al., 1985; Dunlop and Larkins, 1986; Helper and Harden, 1986; Sasaguri et al., 1986; Schnefel et al., 1988; Fleming et
Therefore, we investigated the presence of G-proteins in the guinea pig, namely α-subunits of Gi (Gil, Gβ and Gi3), Gα (Canlon et al., 1991) and of Gβ (Tachibana et al., 1989). Several types of G-proteins have also been described in the organ of Corti of the guinea pig, namely α-subunits of Gi (Gil, Gβ and Gi3), Gα (Canlon et al., 1991) and of Gβ (Tachibana et al., 1992). However, Gα which is most likely associated with the phosphoinositide second messenger system has not yet been demonstrated in the inner ear. In this study, therefore, we investigated the presence of Gα in the CSE and VSE and classified G-proteins using CTX and PTX.

2. Materials and methods

Materials

Fischer-344 rats (3 month-old, male; from Charles River Inc., Kingston, NY) were used in this study. The care and use of animals was reviewed under grant DC-00078 and supervised by the University of Michigan Unit on Laboratory Animal Medicine. Fischer-344 rats (3 month-old, male; from Charles River Inc., Kingston, NY) were used in this study. The care and use of animals was reviewed under grant DC-00078 and supervised by the University of Michigan Unit on Laboratory Animal Medicine. Fischer-344 rats (3 month-old, male; from Charles River Inc., Kingston, NY) were used in this study. The care and use of animals was reviewed under grant DC-00078 and supervised by the University of Michigan Unit on Laboratory Animal Medicine. Fischer-344 rats (3 month-old, male; from Charles River Inc., Kingston, NY) were used in this study. The care and use of animals was reviewed under grant DC-00078 and supervised by the University of Michigan Unit on Laboratory Animal Medicine.

myo-[3H]inositol (specific activity 82 Ci/mmole) and [32P]nicotinamide adenine dinucleotide (NAD; specific activity ~1000 Ci/mmole) were obtained from Amersham Co. (Arlington Heights, IL); Hanks’ balanced salt solution (HBSS) from Gibco BRL Life Technologies Inc. (Gaithersburg, MD). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Tissue preparation

The otic capsules were quickly removed from the temporal bones and placed in incubation buffer at room temperature. The incubation buffer was HBSS with 5 mM sodium N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) gassed with 95% O2/5% CO2 for 30 min; its final pH was adjusted to 7.4 with NaOH and osmolality to 300 ± 2 mOsm with NaCl. The CSE consisting of inner and outer hair cells, supporting cells, basilar membrane and lateral part of the spiral limbus, and VSE consisting of macula utriculi, macula sacculi and cristae ampullares of semicircular canals, were isolated by microdissection, transferred into the incubation buffer and kept at room temperature.

Assay of inositol phosphate release

Details of the incubation procedure were as previously reported (Ogawa and Schacht, 1993). The CSE and VSE were pre-incubated in the absence or presence of CTX or PTX for 2 h at 37°C in 50 μl of incubation buffer and incubated for another 2 h with 1 mM cytidine and 16 μCi myo-[3H]inositol to label phosphoinositide lipids. After removing the myo-[3H]inositol-containing medium, tissues were washed twice with 0.5 ml of non-radioactive buffer. In experiments using permeabilized cells, tissues were treated for 5 min with 50 μl of 50 μg/ml saponin. Tissues were then incubated in 90 μl of buffer with 10 mM LiCl for 10 min, after which 10 μl of buffer with agonists or antagonists were added. Following 30 min of incubation at 37°C, hydrolysis of phosphoinositides was terminated by the addition of 300 μl of chloroform/methanol (1:2, by vol). Prior to separation of the aqueous and chloroform phases, 100 μl of a phytic acid hydrolysate (prepared by heating 4 mg phytic acid in 1 ml of 30 mM acetic acid/65 mM sodium acetate for 48 h at 95°C, quenching with 20 μl of 1 N NaOH and diluting 1:100 with H2O) and 200 μl of chloroform with bovine brain extract (0.2 mg of Sigma type IV/ml) were added as carriers to minimize loss of radiolabeled inositol phosphates and lipids during the isolation procedures.

InsPs in the aqueous phase were separated from myo-inositol on a Dowex-1 formate column as described previously (Berridge et al., 1983; Dean and Beaven, 1989) with minor modifications (Niedzielski et al., 1992; Ogawa and Schacht, 1993). Protein content of each sample was measured by Bradford’s method (Bradford, 1976).

[32P]ADP-ribosylation

The CSE and VSE were incubated for 4 h at 37°C in 50 μl of buffer with or without 1 μg/ml CTX or PTX. Judging from the reproducibility of several ribosylation experiments, this preincubation did not adversely affect the tissues at least not for the purposes of these experiments. Then, tissues were washed twice and homogenized with a microhomogenizer in 10 mM sodium HEPES containing 10 mM MgCl2, 0.2 mM ethylene glycol-bis(β-amino-ethyl ether) N,N,N’,N’-tetra-acetic acid (EGTA), and the protease inhibitors 1 μM leupeptin, 1 μM pepstatin A and 0.2 mM phenylmethane-sulfonyl fluoride (PMF); final pH 7.4. Membrane preparations from CTX-treated cells and their controls were incubated with CTX A-subunit (10 μg/ml), 1 mM GTP, 10 to 20 μCi [32P]NAD, 20 mM dithiothreitol (DTT), 20 mM thymidine and 0.1% Triton X-100 in a total volume of 50 μl at 30°C for 30 min. Membrane preparations from PTX-treated cells and their controls were incubated with activated PTX (10 μg/ml), 1 mM ATP, 5 to 10
μCi [32P]NAD, 20 mM DTT, 20 mM thymidine and 0.1% Triton X-100 in a total volume of 50 μl at 37°C for 30 min. PTX was activated by incubation with 2 mM ATP and 40 mM DTT for 30 min at 37°C (Gill and Woolkalis, 1988). Reactions were terminated by the addition of 500 μl ice-cold buffered saline, and the proteins were pelleted by centrifugation at 12,000 × g for 20 min. The pelleted proteins were solubilized with sodium dodecyl sulfate (SDS) by boiling for 10 min and subjected to electrophoresis on 10% linear gradient SDS-polyacrylamide gels (Laemmli, 1970). Gels were stained for protein with Coomassie Brilliant Blue, dried and autoradiographed using Kodak X-Omat film and an intensifying screen at −70°C for 1 to 4 days. The radioactivity of the bands corresponding to α subunits of G-proteins was measured by scintillation counting.

Statistics
The amount of radioactive InsPs released is expressed as dpm/μg protein. Results are reported as means ± SD. Statistical significance was tested using ANOVA and Fisher's Least Significant Difference.

3. Results

Effects of NaF / AlCl₃ and GTP-γ-S on InsPs release
In order to probe the involvement of G-proteins, the effects of NaF plus AlCl₃ and the non-hydrolysable GTP analog GTP-γ-S on InsPs release were examined in intact and permeabilized tissues, respectively (Fig. 1). Basal release of total InsPs was 4990 ± 1093 dpm/μg protein in the intact CSE and 4925 ± 1189 dpm/μg protein in the intact VSE. Ten mM NaF plus 10 μM AlCl₃ significantly increased InsPs release 1.8-fold in both tissues. In tissues permeabilized with saponin, basal release of InsPs was essentially unchanged with 4949 ± 909 dpm/μg protein in CSE and 5406 ± 780 dpm/μg protein in VSE. The release of InsPs was significantly increased 1.8 and 2.1-fold by 100 μM GTP-γ-S.

Individual InsPs (InsP₁, InsP₂ and InsP₃) were increased 2.2-, 1.6- and 1.5-fold by NaF / AlCl₃ in CSE and 2.2-, 1.1- and 1.2-fold in VSE. GTP-γ-S (200 μM) increased InsP, InsP₂ and InsP₃ release 3.1-, 2.1- and 1.1 fold in permeabilized CSE, and 2.6-, 1.8- and 1.3- in permeabilized VSE, respectively.

Effects of GDP-β-S on agonist-stimulated release of InsPs
Both muscarinic and purinergic P₂, receptors are coupled to PLC in CSE and VSE. To determine the involvement of G-proteins in agonist-stimulated InsPs release, the effect of GDP-β-S was examined in permeabilized tissues. One mM carbamylcholine (CCh) and 200 μM ATP-γ-S enhanced InsPs release to the same extent (Fig. 2) as they did in intact tissues (Ogawa and Schacht, 1993). One hundred μM guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) significantly inhibited carbamylcholine (CCCh)-stimulated release of InsPs by 22% and 30% in CSE and VSE, respectively. Adenosine 5'-O-(3-thiotriphosphate) (ATP-γ-S)-stimulated release of InsPs was also significantly blocked by 33% and 21% in CSE and VSE, respectively.
Table 1

CTX does not affect agonist-stimulated release of InsPs

<table>
<thead>
<tr>
<th>CTX (µg/ml)</th>
<th>CSE CCh (µg/ml)</th>
<th>ATP-γ-S CCh</th>
<th>VSE</th>
<th>ATP-γ-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>192 ± 65</td>
<td>442 ± 97</td>
<td>233 ± 88</td>
<td>375 ± 104</td>
</tr>
<tr>
<td>0.01</td>
<td>180 ± 32</td>
<td>310 ± 124</td>
<td>274 ± 28</td>
<td>356 ± 72</td>
</tr>
<tr>
<td>0.1</td>
<td>172 ± 18</td>
<td>424 ± 127</td>
<td>261 ± 129</td>
<td>381 ± 116</td>
</tr>
<tr>
<td>1</td>
<td>201 ± 68</td>
<td>346 ± 116</td>
<td>284 ± 120</td>
<td>436 ± 240</td>
</tr>
</tbody>
</table>

Intact CSE and VSE were preincubated with CTX (0.01 to 1 µg/ml) for 2 h followed by incubation with 16 µCi myo-[3H]inositol for another 2 h as described in 'Methods'. Then the tissues were incubated with 1 mM carbamylcholine or 200 µM ATP-γ-S for 30 min in the presence of 10 mM LiCl. Each figure is the mean ± SD of 5 independent experiments. Values for CTX-treatment were not different from controls as determined by ANOVA.

Table 2

PTX does not affect agonist-stimulated release of InsPs

<table>
<thead>
<tr>
<th>PTX (µg/ml)</th>
<th>CSE CCh (µg/ml)</th>
<th>ATP-γ-S CCh</th>
<th>VSE</th>
<th>ATP-γ-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>253 ± 62</td>
<td>3977 ± 77</td>
<td>233 ± 77</td>
<td>396 ± 165</td>
</tr>
<tr>
<td>0.01</td>
<td>209 ± 49</td>
<td>467 ± 176</td>
<td>264 ± 80</td>
<td>527 ± 272</td>
</tr>
<tr>
<td>0.1</td>
<td>257 ± 64</td>
<td>433 ± 708</td>
<td>38 ± 55</td>
<td>36 ± 104</td>
</tr>
<tr>
<td>1</td>
<td>249 ± 9</td>
<td>446 ± 140</td>
<td>243 ± 32</td>
<td>442 ± 129</td>
</tr>
</tbody>
</table>

Intact CSE and VSE were preincubated with PTX (0.01 to 1 µg/ml) for 2 h followed by incubation with 16 µCi myo-[3H]inositol for another 2 h as described in 'Methods'. Then the tissues were incubated with 1 mM carbamylcholine or 200 µM ATP-γ-S for 30 min in the presence of 10 mM LiCl. Each figure is the mean ± SD of 5 independent experiments. Values for PTX-treatment were not different from controls as determined by ANOVA.

Accumulation of individual InsP, InsP₂ and InsP₄ induced by CCh was decreased by 20%, 31% and 57% by GDP-β-S in CSE and 19%, 25% and 53% in VSE. Additionally, GDP-β-S blocked the ATP-γ-S stimulated release of InsP, InsP₂ and InsP₄ by 35%, 11% and 36% in permeabilized CSE, and 23%, 5% and 24% in permeabilized VSE.

Effects of cholera and pertussis toxins on agonist-stimulated release of InsPs

In order to classify G-proteins coupled to muscarinic and to purinergic P₂y receptors, tissues were incubated with CTX or PTX for 4 h at 37°C followed by agonist stimulation. The presence of toxins did not affect basal release of InsPs. In addition, neither CCh- nor ATP-γ-S-stimulated release of InsPs was affected by either toxin (Tables 1 and 2). Agonist-stimulated release of individual InsPs was also not affected by these toxins.

ADP-ribosylation by toxins

Two proteins with molecular weights of 52 kDa and 45 kDa were [32P]ADP-ribosylated by the CTX A-subunit in CSE and VSE (Fig. 3a lane 2 and 5). A 40 kDa protein was [32P]ADP-ribosylated by activated PTX in these tissues (Fig. 3b lane 2 and 5). No radioactivity was incorporated into the protein bands when the membrane fraction was incubated with [32P]NAD without toxins (Fig. 3a,b; lane 1 and 4). Under the same conditions as the assay of inositol phosphate hydrolysis, [32P]ADP-ribosylation of the 52 kDa and 45 kDa proteins was decreased by 59 ± 12% in CSE and by 55 ± 19% in VSE (n = 5) by pretreatment with 1 µg/ml CTX (Fig. 3a, lane 3 and 6). [32P]ADP-ribosylation of the 40 kDa substrate was reduced by 70 ± 15% in CSE and by 73 ± 14% in VSE (n = 5) by pretreatment with 1 µg/ml PTX (Fig. 3b, lane 3 and 6).

(a) CTX

(b) PTX

![Fig. 3. SDS-PAGE separation of membrane proteins [32P]ADP-ribosylated by CTX (Fig. 3a) or PTX (Fig. 3b). CSE (lanes 1, 2 and 3) and VSE (lanes 4, 5 and 6) were pre-incubated with or without CTX or PTX for 4 h at 37°C. Then, the membrane fractions were [32P]ADP-ribosylated in the presence of the respective toxin as described in 'Methods'. Lanes 1 and 4 represent controls incubated without CTX A-subunit or activated PTX; lanes 2 and 5 are from pre-incubations without toxin; lanes 3 and 6 from pre-incubations with toxin.](image-url)
4. Discussion

The present study clearly demonstrates the involvement of G-proteins in the phosphoinositide second messenger system both in the cochlear and vestibular sensory epithelia. The type of G-protein, however, is unlike any of those characterized in the inner ear so far.

In support of the involvement of G-proteins in the regulation of PLC activity, GTP-γ-S stimulates InsPs release. GTP itself is not an effective activator of G-proteins, because it is easily hydrolyzed to GDP. The non-hydrolyzable analog GTP-γ-S, however, has been shown to enhance InsPs release in various organs (Litosch et al., 1985; Cockcroft and Gompert, 1985). The implication that PLC activation is regulated by G-proteins in the CSE and VSE is corroborated by the fact that NaF and AlCl₃ also enhanced InsPs release. The combination of these compounds, presumably acting as fluoroaluminate (AlF₃⁻), mimics the γ-phosphate group of GTP and activates G-proteins in intact cells (Sternweis and Gilman, 1982; Katada et al., 1984; Cockcroft and Taylor, 1987, Marc et al., 1988; Fischer et al., 1993). Furthermore, individual InsPs including InsP₃, were also increased by GTP-γ-S or AlF₃⁻, suggesting that the hydrolysis of PtdInsP₂ was a main pathway to release InsPs.

In a second line of evidence, the agonist-mediated release of InsPs was significantly blocked by GDP-β-S. This GDP analog inhibits the activation of G-protein, and its effect was evident on the activation of both muscarinic and purinergic P₃ receptors in the CSE and VSE. The sum of these results provides convincing evidence that G-proteins are coupled to the phosphoinositide second messenger system in CSE and VSE. This general evidence, however, does not indicate which type of G-protein is involved.

Both CTX- and PTX-sensitive G-proteins are found in the cochlea. The toxins are specific probes for G-proteins as they catalyze the irreversible covalent addition of an ADP-ribosyl group from intracellular NAD⁺ to the α subunit of Gα (CTX) or Gγ (PTX). Thus, the ADP-ribosylation of proteins with molecular weights of 52 kDa and 45 kDa catalyzed by CTX is consistent with the presence of Gα₁ or Gα₂ and Gα₃ or Gα₄, respectively. Proteins with a molecular weight of 40 kDa which are ADP-ribosylated by PTX are considered to be Gγ₁ or Gγ₂ (Kaziro et al., 1991). These findings are in agreement with the demonstration of α-subunits of inhibitory G-proteins (Gα₁, Gα₂ and Gγ), other G-protein (Gγ) and stimulatory G-protein (Gγ) in the organ of Corti of guinea pig (Canlon et al., 1991; Tachibana et al., 1992). None of these toxin-sensitive G-proteins, however, seems involved in the coupling of cochlear and vestibular muscarinic and purinergic receptors to phospholipase C.

This finding in CSE and VSE is in agreement with the characterization of PLC-coupled G-proteins in most tissues. G-proteins associated with muscarinic receptors have generally been reported to be CTX- and PTX-insensitive (Masters et al., 1985; Dunlop and Larkins, 1986; Helper and Harden, 1986; Sasaguri et al., 1986; Schnefel et al., 1988; Fleming et al., 1989) or at best incompletely blocked at high PTX concentrations (10 μg/ml; Met et al., 1988). In contrast, patch-clamping studies of isolated cochlear hair cells inferred that acetylcholine-stimulation of the inositol phosphate cascade was mediated through a PTX-sensitive G-protein (Kakehata et al., 1992; Kakehata et al., 1993). However, the acetylcholine action was measured as an activation of a calcium-activated K⁺-channel, not InsPs release directly. In addition, this type of response is blocked by both atropine and d-tubocurare (Housley and Ashmore, 1991) in contrast to the selective inhibition of InsPs release by atropine only (Niedzielski et al., 1992; Ogawa and Schacht, 1993). Thus, G-proteins related to a calcium-activated K⁺-channel in outer hair cells may be different from G-proteins coupled to the phosphoinositide second messenger system in the CSE and VSE.

The types of G-proteins involved in purinergic signal transduction remain to be established. Partial inhibition of InsPs release by PTX at purinergic receptors has been reported in some tissues (Haggblad and Heilbronn, 1988; Okajima et al., 1989; Dubyak et al., 1990; Nanoff et al., 1990; Yamada et al., 1992). In these cases, InsPs release by low concentration of purinergic agonists was blocked by PTX, while the toxin produced no or only partial inhibition at high agonist concentrations. This is consistent with our study in which InsPs release induced by a high concentration of ATP-γ-S was unaffected by toxins.

Finally, the ineffectiveness of the toxin action can only be used as an argument if it is assured that the toxin actually entered the intact tissues of the CSE and VSE. ADP-ribosylation demonstrating the presence of G-proteins was conducted in tissue homogenates, and thus did not indicate whether the toxin can enter the cells under the experimental conditions of InsPs release. Two points can be made. First, the conditions used here for both CTX and PTX have been shown to be effective on other cell types. For example, 10 nM CTX incubated with mouse neuroblastoma cells was internalized with a half-life of 2 h (Fishman, 1982). In rat C6 glioma cells, the half-maximal effective concentration for a 2 h incubation was 100 ng PTX/ml (Katada et al., 1982). Second, the prior incubation of intact tissues with toxins in our experiments led to a significant decrease of ADP-ribosylation, indicating that the toxin was able to enter the cells.

In summary, G-proteins are key components in the regulation of intracellular signal transduction. We have
previously postulated that the effenter system regulates or modulates hair cell functions through the phosphoinosside second messenger cascade. We can now conclude that muscarinic and purinergic receptors in CSE and VSE are coupled to phospholipase C through \(G_{\alpha}\)-like G-proteins. In order to elucidate the precise role of Gp in signal transduction in the inner ear we need further characterization of these proteins by molecular biological approaches and studies of their involvement in hair cell physiology and pathophysiology.

5. Acknowledgements

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6. References


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